

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/82, 15/12, A01H 5/00		A1	(11) International Publication Number: WO 96/40949 (43) International Publication Date: 19 December 1996 (19.12.96)
(21) International Application Number: PCT/US96/08583 (22) International Filing Date: 6 June 1996 (06.06.96) (30) Priority Data: 08/475,582 7 June 1995 (07.06.95) US		(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(71) Applicant: PIONEER HI-BRED INTERNATIONAL, INC. [US/US]; 700 Capital Square, 400 Locust Street, Des Moines, IA 50309 (US). (72) Inventors: HOWARD, John, A.; 2976 N.W. 132nd Street, Des Moines, IA 50325 (US). ALBERTSON, Marc, C.; 1930 S. 60th Street, West Des Moines, IA 50266 (US). (74) Agents: BENT, Stephen, A. et al.; Foley & Lardner, Suite 500, 3000 K Street, N.W., Washington, DC 20007-5109 (US).			
(54) Title: INDUCTION OF MALE STERILITY IN PLANTS BY EXPRESSION OF HIGH LEVELS OF AVIDIN (57) Abstract <p>Male-sterile plants can be produced by increasing the endogenous concentration of avidin in the plant tissues. This effect can be achieved by producing transgenic plants containing an expression vector in which a promoter is operably linked to a DNA sequence encoding avidin. Methods for restoring male fertility are also disclosed.</p> <p><i>21 - as of 6/6 22 - 1st SP 10/97</i></p>			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LJ	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

**INDUCTION OF MALE STERILITY IN PLANTS BY
EXPRESSION OF HIGH LEVELS OF AVIDIN**

BACKGROUND OF THE INVENTION

The present invention relates to a method for controlling fertility of plants using DNA molecules that encode avidin. In particular, this invention is directed to methods for producing transgenic, male-sterile plants which express avidin. Moreover, this invention is directed to methods for restoring male fertility in the progeny of male-sterile plants.

Control of pollen fertility is essential in hybrid crop production. See, for example, J. M. Poehlman, BREEDING FIELD CROPS, 3rd ed. (Van Nostrand and Reinhold, New York, NY, 1987), which is herein incorporated by reference. In hybrid maize production, for example, control of pollen fertility is typically accomplished by physically removing the male inflorescence, or tassel, prior to pollen shed. Manual detasseling is highly labor intensive. Although mechanical detasseling is less labor intensive than manual detasseling, it is less reliable and requires subsequent examination of the crop and possibly, remedial manual detasseling. Both methods of detasseling cause a loss of female parent yield.

Most major crop plants of interest, however, have both functional male and female organs within the same flower; therefore, emasculation is not a simple procedure. While it is possible to remove by hand the pollen-forming organs before pollen is shed, this form of hybrid production is extremely labor intensive and expensive.

Pollen fertility also can be controlled by applying a foliar spray having male gametocidal properties. Cross et al., *Sex Plant Reprod.* 4: 235 (1991). For example, the application of ethrel to anthers results in additional pollen mitoses in wheat, degeneration of tapetal cells in barley, and malformed or aborted pollen in *Eragrostis*. Bennet et al., *Nature* 240: 566 (1972), Colhoun et al., *Plant Cell Environ.* 6: 21 (1983); Berthe

et al., *Crop Sci.* 18: 35 (1978). However, the chemical approach is labor intensive, and presents potential problems with the toxicity of chemicals introduced into the environment.

5 In addition, commercial production of hybrid seed by use of gametocides is limited by the expense and availability of the chemicals, as well as by the reliability and length of action of the applications. A serious limitation of gametocides is that they have
10 phytotoxic effects, the severity of which are dependent on genotype. Other limitations include that these chemicals may not be effective for crops with an extended flowering period because new flowers produced may not be affected. Consequently, repeated application of
15 chemicals is required.

Many current commercial hybrid seed production systems for field crops rely on a genetic means of pollination control. Plants that are used as females either fail to shed pollen, produce pollen that is
20 biochemically unable to effect self-fertilization, or fail to make pollen. Plants that are unable to self-fertilize are said to be "self-incompatible." Difficulties associated with the use of a self-incompatibility system include availability and
25 propagation of the self-incompatible female line, and stability of the self-compatibility. In some instances, self-incompatibility can be overcome chemically, or immature buds can be pollinated by hand before the biochemical mechanism that blocks pollen is activated.
30 Self-incompatible systems that can be deactivated are often very vulnerable to stressful climatic conditions that break or reduce the effectiveness of the biochemical block to self-pollination.

35 Of more widespread interest for commercial seed production are genetic pollen-control-based systems causing male sterility. These systems are of two general types: (1) nuclear male sterility, which is the failure of pollen formation because of mutations in one or more

nuclear genes or (2) cytoplasmic-genetic male sterility, commonly referred to as "cytoplasmic male sterility" (CMS), in which pollen formation is blocked or aborted because of an alteration in a cytoplasmic organelle, which generally is the mitochondria.

Nuclear sterility can be either dominant or recessive. Dominant sterility can only be used for hybrid seed formation if vegetative or clonal propagation of the female line is possible. Recessive sterility can be used if sterile and fertile plants are easily discriminated. Commercial utility of dominant and recessive sterility systems is limited, however, by the expense of clonal propagation and roguing the female rows of self-fertile plants, respectively.

Although there are hybridization schemes involving the use of CMS, there are limitations to its commercial value. One example of a CMS system is a specific mutation in the cytoplasmically located mitochondria which can, when in the proper nuclear background, lead to the failure of mature pollen formation. In some instances, the nuclear background can compensate for the cytoplasmic mutation and normal pollen formation occurs. Specific nuclear "restorer genes" allow pollen formation in plants with CMS mitochondria. Generally, the use of CMS for commercial seed production involves the use of three breeding lines: a male-sterile line (female parent), a maintainer line which is isogenic to the male-sterile line but contains fully functional mitochondria, and a male parent line. The male parent line may or may not carry the specific restorer genes in the cytoplasm.

For crops such as vegetables for which seed recovery from the hybrid is unimportant, a CMS system can be used without restoration. For crops for which the fruit or seed of the hybrid is the commercial product, the fertility of the hybrid seed must be restored by specific restorer genes in the male parent or the male-sterile hybrid must be pollinated. Pollination of non-restored

hybrids can be achieved by including a small percentage of male fertile plants to effect pollination. In most species, the CMS trait is inherited maternally, since all cytoplasmic organelles are inherited from the egg cell
5 only.

CMS systems possess limitations that can preclude them as a sole solution to production of male sterile plants. For example, one particular CMS type in maize (T-cytoplasm) confers sensitivity to the toxin produced
10 by infection by a particular fungus. Although still used for a number of crops, CMS systems may break down under certain environmental conditions.

It is apparent, therefore, that a means to control pollen production by other than manual, mechanical,
15 chemical and traditional genetic methods is greatly to be desired.

SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to provide a method to produce male-sterile plants that
20 are rendered sterile by heterologous expression of avidin.

It is a further object of this invention to provide a chimeric gene comprising a DNA sequence that encodes avidin which is operably linked to a plant promoter sequence.
25

It is a further object of the invention to produce a method of reversing male sterility caused by expression of avidin.

These and other objects are achieved, in accordance
30 with one embodiment of the present invention, by the provision of an isolated DNA molecule comprising a nucleotide sequence encoding avidin operably linked to a plant promoter sequence. In a preferred embodiment the isolated DNA sequence is contained in an expression vector. In another preferred embodiment the plant promoter sequence is a constitutive promoter, and in
35 another preferred embodiment the constitutive promoter is a ubiquitin promoter.

In accordance with another embodiment of the invention there is provided a transgenic plant, in which the plant comprises a heterologous nucleotide sequence comprising an avidin gene, and wherein the heterologous nucleotide sequence increases the concentration of avidin in the tissues of the plant, causing male sterility. In preferred embodiments the transgenic plant is a corn plant, a soybean plant, or a sunflower plant. In another preferred embodiment the heterologous DNA molecule further comprises a constitutive promoter sequence, which in a preferred embodiment is a ubiquitin promoter sequence.

In accordance with still another aspect of the invention there is provided a method of producing a transgenic male-sterile plant, comprising introducing into plant cells an expression vector comprising a promoter and an avidin gene, wherein the promoter controls the expression of the avidin gene, and where the avidin gene expression causes male sterility. In a preferred embodiment a transgenic plant is regenerated from the plant cells. In another preferred embodiment the promoter comprises a ubiquitin promoter or an inducible promoter.

In accordance with still another aspect of the invention there is provided a method of using an avidin gene to produce a male-fertile hybrid plant, comprising producing a first parent male-sterile plant comprising a DNA molecule as described above, in which the expression of avidin causes male sterility, producing a second transgenic parent plant expressing a second foreign gene, and cross-fertilizing the first parent with the second parent to produce a hybrid plant that expresses the second foreign gene, and in which the product of the second foreign gene reduces expression of avidin in the hybrid plant, thereby producing a male-fertile hybrid plant.

In a preferred embodiment of this aspect of the invention the second foreign gene is selected from the

group consisting of an antisense gene, a ribozyme gene and an external guide sequence gene. In another preferred embodiment the antisense gene comprises avidin mRNA sequences, which in a further preferred embodiment
5 is under the control of an inducible promoter. In still another preferred embodiment the ribozyme gene comprises avidin mRNA sequences. In yet another preferred embodiment the external guide sequence gene comprises avidin mRNA sequences. In still further preferred
10 embodiments the DNA molecule of the first parent plant further comprises a LexA operator which is operatively linked to said promoter, wherein the second foreign gene is the LexA repressor gene. In another preferred embodiment the promoter sequence is an anther-specific
15 promoter sequence comprising an anther box selected from the group consisting of the nucleotide sequence of SEQ ID NO:1; the nucleotide sequence of SEQ ID NO:2; the nucleotide sequence of SEQ ID NO:3; and a functional fragment thereof.

20 In still another preferred embodiment of this aspect of the invention the anther box has the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3, and the anther-specific promoter further comprises a core promoter selected from the group consisting of: CaMV 35S
25 core promoter; the nucleotide sequence of SEQ ID NO:4; the nucleotide sequence of SEQ ID NO:5; the nucleotide sequence of SEQ ID NO:6; and functional fragments thereof.

30 In accordance with a still further aspect of the invention there is provided a method for restoring fertility in a plant rendered male sterile by expression of avidin, comprising spraying the plant with a solution of biotin.

BRIEF DESCRIPTION OF THE DRAWINGS

35 Figure 1 shows the avidin-encoding plasmid PHI5168.

Figure 2 shows the plasmid PHI610 encoding the bar gene.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS**1. Definitions**

In the description that follows, a number of terms are used extensively. The following definitions are 5 provided to facilitate understanding of the invention.

A structural gene is a DNA sequence that is transcribed into messenger RNA (mRNA) which is then translated into a sequence of amino acids characteristic of a specific polypeptide.

10 A foreign gene refers in the present description to a DNA sequence that is operably linked to at least one heterologous regulatory element. For example, any gene other than the maize 5126 structural gene is considered to be a foreign gene if the expression of that gene is 15 controlled by an anther-specific regulatory element of the 5126 gene.

A promoter is a DNA sequence that directs the transcription of a gene, such as a structural gene, an antisense gene, a ribozyme gene or an external guide 20 sequence gene. Typically, a promoter is located in the 5' region of a gene, proximal to the transcriptional start site. If a promoter is an inducible promoter, then the rate of transcription increases in response to an inducing agent. In contrast, the rate of transcription 25 is not regulated by an inducing agent if the promoter is a constitutive promoter. A plant promoter is a promoter sequence that will direct the transcription of a gene in plant tissue.

A core promoter contains essential nucleotide 30 sequences for promoter function, including the TATA box and start of transcription. By this definition, a core promoter may or may not have detectable activity in the absence of specific sequences that may enhance the activity or confer tissue specific activity. For 35 example, the SGB6 core promoter consists of about 38 nucleotides 5'-ward of the transcriptional start site of the SGB6 gene, while the Cauliflower Mosaic Virus (CaMV)

35S core promoter consists of about 33 nucleotides 5'-ward of the transcriptional start site of the 35S genome.

A tissue-specific promoter is a DNA sequence that, when operably linked to a gene, directs a higher level of transcription of that gene in a specific tissue than in some or all other tissues in an organism. For example, 5 an anther-specific promoter is a DNA sequence that directs a higher level of transcription of an associated gene in plant anther tissue. The SGB6 anther-specific 10 promoter, for example, can direct the expression of a foreign gene in anther tissue, but not in root tissue or coleoptile tissue.

As used herein, an "anther-specific promoter" comprises two functional elements: an "anther box" and a 15 core promoter. A particular anther box may possess the functional characteristics of a classic enhancer. The combination of an anther box and a core promoter can stimulate gene expression to a greater extent than a core promoter alone. This is true even in the case of a 20 chimeric anther-specific promoter containing an anther box and a core promoter derived from different genes. Such chimeric anther-specific regulatory elements are described below.

An operator is a DNA molecule that is located 5'-ward 25 of a gene and that contains a nucleotide sequence which is recognized and bound by a repressor protein. The binding of a repressor protein with its cognate operator results in the inhibition of the transcription of the gene. For example, the LexA gene encodes a repressor 30 protein that binds to the LexA operator.

An isolated DNA molecule is a fragment of DNA that has been separated from the DNA of an organism. For example, a cloned DNA molecule encoding an avidin gene is 35 an isolated DNA molecule. Another example of an isolated DNA molecule is a chemically-synthesized DNA molecule, or enzymatically-produced cDNA, that is not integrated in the genomic DNA of an organism.

An enhancer is a DNA regulatory element that can increase the efficiency of transcription.

5 Complementary DNA (cDNA) is a single-stranded DNA molecule that is formed from an mRNA template by the enzyme reverse transcriptase. Typically, a primer complementary to portions of mRNA is employed for the initiation of reverse transcription. Those skilled in the art also use the term "cDNA" to refer to a double-stranded DNA molecule consisting of such a single-stranded DNA molecule and its complementary DNA strand.

10 The term expression refers to the biosynthesis of a gene product. For example, in the case of a structural gene, expression involves transcription of the structural gene into mRNA and the translation of mRNA into one or more polypeptides.

15 A cloning vector is a DNA molecule, such as a plasmid, cosmid, or bacteriophage, that has the capability of replicating autonomously in a host cell. Cloning vectors typically contain one or a small number 20 of restriction endonuclease recognition sites at which foreign DNA sequences can be inserted in a determinable fashion without loss of an essential biological function of the vector, as well as a marker gene that is suitable for use in the identification and selection of cells 25 transformed with the cloning vector. Marker genes typically include genes that provide tetracycline resistance or ampicillin resistance.

30 An expression vector is a DNA molecule comprising a gene that is expressed in a host cell. Typically, gene expression is placed under the control of certain regulatory elements, including constitutive or inducible promoters, tissue-specific regulatory elements, and enhancers. Such a gene is said to be "operably linked to" the regulatory elements.

35 A recombinant host may be any prokaryotic or eukaryotic cell that contains either a cloning vector or expression vector. This term also includes those prokaryotic or eukaryotic cells that have been

genetically engineered to contain the cloned gene(s) in the chromosome or genome of the host cell.

A transgenic plant is a plant having one or more plant cells that contain a foreign gene.

5 In eukaryotes, RNA polymerase II catalyzes the transcription of a structural gene to produce mRNA. A DNA molecule can be designed to contain an RNA polymerase II template in which the RNA transcript has a sequence that is complementary to that of a specific mRNA. The 10 RNA transcript is termed an antisense RNA and a DNA sequence that encodes the antisense RNA is termed an antisense gene. Antisense RNA molecules are capable of binding to mRNA molecules, resulting in an inhibition of mRNA translation.

15 A ribozyme is an RNA molecule that contains a catalytic center. The term includes RNA enzymes, self-splicing RNAs, and self-cleaving RNAs. A DNA sequence that encodes a ribozyme is termed a ribozyme gene.

20 An external guide sequence is an RNA molecule that directs the endogenous ribozyme, RNase P, to a particular species of intracellular mRNA, resulting in the cleavage of the mRNA by RNase P. A DNA sequence that encodes an external guide sequence is termed an external guide sequence gene.

25 2. Overview

The present invention provides methods for generating male sterile plants by preparing transgenic plants that express avidin. The avidin can be expressed constitutively, in a non-tissue specific manner, or can be expressed in an anther-specific manner. Methods for restoring male fertility are also provided.

An avidin gene is isolated and inserted into an expression vector suitable for introduction into plant tissue. The expression vector further comprises a 35 promoter which may be a constitutive promoter, a non-tissue specific promoter, such as the ubiquitin promoter, a tissue specific promoter such as an anther-specific promoter, or an inducible promoter. The expression

vector is introduced into plant tissue by standard methods and transgenic plants are selected and propagated. The transgenic plants that express avidin are male sterile. Male fertility can be restored by 5 coexpression in the transgenic plants of a second gene that inhibits transcription of the avidin gene, or inhibits translation of avidin mRNA. Alternatively, male fertility can be restored by spraying developing plants with solutions of biotin.

10 **3. Isolation of DNA Molecules that Encode Avidin**

Nucleotide sequences encoding avidin can be isolated by known methods. For example, a cDNA encoding chicken egg white avidin can be isolated from a chicken oviduct cDNA library by the methods described by Gope et al., 15 *Nucleic Acids Res.*, 15:3595 (1987), which is incorporated herein by reference.

Alternatively, genomic clones of the avidin gene can be obtained from genomic DNA of organisms that are known to produce avidin. For example, a chicken avidin gene 20 can be cloned from chicken genomic DNA by the methods described in Keinanen et al., *Eur. J. Biochem.* 220: 615 (1994).

Avidin genes can also be isolated using the polymerase chain reaction (PCR) using standard methods. 25 See Erlich, *PCR TECHNOLOGY: PRINCIPLES AND APPLICATIONS FOR DNA AMPLIFICATION* (Stockton Press, NY, 1989) and Innis et al., *PCR PROTOCOLS: A GUIDE TO METHODS AND APPLICATIONS* (Academic Press, San Diego, 1990). Methods for PCR amplification of long nucleotide sequences, such 30 as the ELONGASE™ system (Life Technologies, Inc., Gaithersburg, MD) can also be used for obtaining longer nucleotide sequences, such as genomic clones encoding avidin. PCR primers complementary to the 5' and 3' termini of known avidin gene sequences can be synthesized 35 using commercial oligonucleotide synthesizers, such as those supplied by Applied Biosystems (Foster City, CA). In a preferred embodiment, the primers include additional nucleotide sequences containing restriction endonuclease

cleavage sites. The presence of such sites allows for the directional cloning of PCR products into suitable cloning vectors after treatment with an appropriate restriction enzyme. See Finney, "Molecular Cloning of 5 PCR Products" in CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Ausubel et al. Eds. (John Wiley & Sons, New York, 1987) p. 15.7.1.

Template DNA for the PCR can either be cDNA or genomic DNA, and can be prepared from an appropriate 10 organism using methods well known in the art. Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL, Second Edition, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY 1989). Genomic DNA can be directly prepared from avian, reptilian, or amphibian tissue using 15 commercials reagents, such as Triazol™ (Life Technologies, Inc., Gaithersburg, MD). Alternatively, cDNA encoding avidin can be prepared using mRNA extracted from chicken oviduct tissue using a commercially available kit (Pharmacia, Piscataway, NJ). The mRNA 20 preparation is used as a template for cDNA synthesis using poly(dT) or random hexamer primers by standard techniques. See Sambrook et al., supra. cDNA synthesis is then carried out using a commercially available kit 25 (Pharmacia), and the cDNA used directly for PCR using the method of Saiki et al., *Science* 239: 487 (1988).

Genomic DNA or cDNA fragments isolated by the methods described above can be inserted into a vector, such as a bacteriophage or cosmid vector, in accordance with conventional techniques, such as the use of restriction 30 enzyme digestion to provide appropriate termini, the use of alkaline phosphatase treatment to avoid undesirable joining of DNA molecules, and ligation with appropriate ligases. Techniques for manipulation of such vectors are disclosed by Ausubel et al. (eds.), CURRENT PROTOCOLS IN 35 MOLECULAR BIOLOGY, pages 3.0.5-3.17.5 (1990) ["Ausubel"].

Alternatively, nucleotide sequences encoding avidin can be obtained by synthesizing DNA molecules encoding avidin using mutually priming long oligonucleotides.

See, for example, Ausubel at pages 8.2.8 to 8.2.13. Also, see Wosnick et al., *Gene* 60:115 (1987). Current techniques using the polymerase chain reaction provide the ability to synthesize genes as large as 1.8 kilobases
5 in length. Adang et al., *Plant Molec. Biol.* 21: 1131 (1993); Bambot et al., *PCR Methods and Applications* 2: 266 (1993). The nucleotide sequence of a synthesized avidin gene can be based upon any known avidin-encoding DNA molecule. See, for example, Gope et al., *supra*.

10 These clones can be analyzed using a variety of standard techniques such as restriction analysis, Southern analysis, primer extension analysis, and DNA sequence analysis. Primer extension analysis or S1 nuclease protection analysis, for example, can be used to
15 localize the putative start site of transcription of the cloned gene. Ausubel at pages 4.8.1-4.8.5; Walmsley et al., "Quantitative and Qualitative Analysis of Exogenous Gene Expression by the S1 Nuclease Protection Assay," in METHODS IN MOLECULAR BIOLOGY, VOL. 7: GENE TRANSFER AND
20 EXPRESSION PROTOCOLS, Murray (ed.), pages 271-281 (Humana Press Inc. 1991).

4. Cloning of avidin genes into an expression vector and preparation of transgenic plants

Once an avidin gene has been isolated it is placed
25 into an expression vector by standard methods. See Sambrook et al., *supra*. The selection of an appropriate expression vector will depend upon the method of introducing the expression vector into host cells. Typically, an expression vector contains: (1) prokaryotic
30 DNA elements coding for a bacterial replication origin and an antibiotic resistance gene to provide for the growth and selection of the expression vector in the bacterial host; (2) a cloning site for insertion of an exogenous DNA sequence, for example an avidin gene; (3)
35 eukaryotic DNA elements that control initiation of transcription of the exogenous gene, such as a promoter; (4) DNA elements that control the processing of transcripts, such as a transcription

termination/polyadenylation sequence; and (5) a gene encoding a marker protein (e.g., a reporter gene), wherein the gene is operably linked to the DNA elements that control transcription initiation. General 5 descriptions of plant expression vectors and reporter genes can be found in Gruber et al., "Vectors for Plant Transformation," in METHODS IN PLANT MOLECULAR BIOLOGY AND BIOTECHNOLOGY, Glick et al. (eds.), pages 89-119 (CRC Press, 1993).

10 In a preferred embodiment of the present invention a plant ubiquitin promoter system is used. Plant ubiquitin promoters are well known in the art. See, for example, European Patent Application 0 342 926, which is incorporated herein by reference. The ubiquitin promoter 15 is a constitutive promoter.

20 In another preferred embodiment of the invention, an inducible promoter is used to allow inducible regulation of the expression of avidin. An example of such an inducible promoter is the glutathione S-transferase 25 system in maize. See Wiegand et al., *Plant Mol. Biol.* 7:235 (1986). This method also allows for reversible induction of male sterility. Thus, expression of avidin and concomitant male sterility, can be controlled as desired by activation of the promoter. When the promoter 30 is not activated, avidin is not expressed, and the plants are male fertile.

35 Expression vectors containing an avidin gene can be introduced into protoplasts, or into intact tissues, such as immature embryos and meristems, or into callus cultures, or into isolated cells. Preferably, expression vectors are introduced into intact tissues. General methods of culturing plant tissues are provided, for example, by Miki et al., "Procedures for Introducing Foreign DNA into Plants," in METHODS IN PLANT MOLECULAR BIOLOGY AND BIOTECHNOLOGY, Glick et al. (eds.), pages 67-88 (CRC Press, 1993), and by Phillips et al., "Cell/Tissue Culture and In Vitro Manipulation," in CORN AND CORN IMPROVEMENT, 3rd Edition, Sprague et al. (eds.),

pages 345-387 (American Society of Agronomy, Inc. et al. 1988).

Methods of introducing expression vectors into plant tissue include the direct infection or co-cultivation of 5 plant tissue with *Agrobacterium tumefaciens*. Horsch et al., *Science* 227:1229 (1985). Preferably, a disarmed Ti-plasmid is used as a vector for foreign DNA sequences. Transformation can be performed using procedures described, for example, in European patent application 10 Nos. 116 718 (1984) and 270 822 (1988). Preferred Ti-plasmid vectors contain the foreign DNA sequence between the border sequences, or at least located upstream of the right border sequence.

Other types of vectors can be used for transforming 15 plant cells using procedures such as direct gene transfer (see, for example, PCT application WO 85/01856 and European application 275 069), in vitro protoplast transformation (for example, U.S. patent No. 4,684,611), plant virus-mediated transformation (for example, 20 European application No. 067 553 and U.S. patent No. 4,407,956), and liposome-mediated transformation (for example, U.S. patent No. 4,536,475). Suitable methods for corn transformation are provided by Fromm et al., *Bio/Technology* 8: 833 (1990), and by Gordon-Kamm et al., 25 *The Plant Cell* 2: 603 (1990). Standard methods for the transformation of rice are described by Christou et al., *Trends in Biotechnology* 10: 239 (1992), and by Lee et al., *Proc. Nat'l Acad. Sci. USA* 88: 6389 (1991). Wheat can be transformed using methods that are similar to the 30 techniques for transforming corn or rice. Furthermore, Casas et al., *Proc. Nat'l Acad. Sci. USA* 90: 11212 (1993), describe a method for transforming sorghum, while Wan et al., *Plant Physiol.* 104: 37 (1994), describe a method for transforming barley.

35 In general, direct transfer methods are preferred for the transformation of a monocotyledonous plant, particularly a cereal such as rice, corn, sorghum, barley or wheat. Suitable direct transfer methods include

microprojectile-mediated delivery, DNA injection, electroporation, and the like. See, for example, Gruber et al., *supra*, Miki et al., *supra*, and Klein et al., *Bio/Technology* 10: 268 (1992). More preferably, 5 expression vectors are introduced into tissues of a monocotyledonous plant using microprojectile-mediated delivery with a biolistic device.

5. Regulation of Male Fertility

A. Production of Male-Sterile Plants

10 To induce male sterility pursuant to the present invention, an expression vector is constructed in which a DNA sequence encoding avidin is operably linked to DNA sequences that regulate gene transcription in plant tissue. The general requirements of an expression vector 15 are described above. In order to achieve male sterility by expression of avidin it is preferred that avidin be not merely expressed in a transient manner, but that the expression vector is introduced into plant embryonic tissue in such a manner that avidin will be expressed at 20 a later stage of development in the adult plant. For example, mitotic stability can be achieved using plant viral vectors that provide epichromosomal replication.

A preferred method of obtaining mitotic stability is provided by the integration of expression vector 25 sequences into the host chromosome. Such mitotic stability can be provided by the microprojectile delivery of an expression vector to plant tissue, or by using other standard methods as described above. See, for example, Fromm et al., *Bio/Technology* 8: 833 (1990), 30 Gordon-Kamm et al., *The Plant Cell* 2: 603 (1990), and Walters et al., *Plant Molec. Biol.* 18: 189 (1992).

Transcription of the avidin gene after introduction 35 into plant tissue is preferably controlled by a constitutive promoter that non-specifically stimulates gene expression in plant tissue. An example of a constitutive promoter suitable for this purpose is the ubiquitin promoter, as described *supra*.

Transcription of the avidin gene may also be controlled by a promoter which stimulates gene expression in a tissue-specific manner. A particularly preferred anther-specific promoter is the 5126 promoter which was 5 isolated from the maize inbred B73 line. The 5126 promoter stimulates the expression of a foreign gene from quartet to early uninucleate microspore-stage anthers.

Another suitable anther-specific promoter is the SGB6 promoter, which also was isolated from the B73 line. The 10 SGB6 promoter can induce expression of a foreign gene in anther tapetal cells from the quartet stage to the mid-uninucleate stage of microspore development.

Alternatively, anther-specific gene expression can be provided using a combination of an anther box and a 15 core promoter. A particularly preferred anther box is a 5126 anther box which comprises the following nucleotide sequence:

5' GCGGCCGCGG ATCCGCTCAT CTGCTCGGTA CCAACCAGCC
CGCCGGCGCC TAGGCGAGTA GACGAGCCAT GGTTGGTCGG
20 TTTCTTATTAA CCATGCACAG ATCT 3' [SEQ ID NO:1].
AAAGGATAAT GGTACGTGTC TAGA

Another suitable anther box resides within a 94 base pair DNA fragment defined by nucleotides 583 to 490 upstream of the SGB6 start site of transcription. The 25 nucleotide sequence of the 94 base pair SGB6 anther box is:

(-583) ACAGTTCACT AGATATGCAT GATCTTTAAC AATTGCTGCT
TGTCAAGTGA TCTATACGTA CTAGAAATTG TTAACGACGA
30 GGATTGTGCG GTTTCTTTG GCACAAATGG CATGAACAGA
CCTAACACGC CAAAGAAAAC CGTGTTCACC GTACTTGTCT
GTAATCCGGG ACGC (-490) [SEQ ID NO: 2].
CATTAGGCC CGCG

Alternatively, a suitable anther box is obtained from the maize G9 promoter, which stimulates gene expression

during the meiotic to quartet stages of development. The G9 anther box comprises the following nucleotide sequence:

5' GCGGCCGCGG ATCCTGGCTG GATGAAACCG ATGCGAGAGA
CGCCGGCGCC TAGGACCGAC CTACTTTGGC TACGCTCTCT

AGAAAAAAA ATTGTTGCAT GTAGTTGGCG CCTGTCACCC
TCTTTTTTT TAACAACGTA CATCAACCGC GGACAGTGCG

AACCAAACCA GTAGTTGAGG CACGCCCTGT TTGCTCACGA
TTGGTTGGT CATCAACTCC GTGCGGGACA AACGAGTGCT

10 TCACGAACGT AGATCT 3' [SEQ ID NO:3].
AGTGCTTGCA TCTAGA

The 5126, SGB6 and G9 anther boxes can be obtained by synthesizing oligonucleotides, as described above. Those of skill in the art will appreciate that deletion analysis can be performed to localize one or more additional anther-specific regulatory sequences within the disclosed anther boxes. Such "functional fragments" of the anther boxes also can be used to regulate avidin gene expression in an anther-specific manner.

20 Preferred core promoters are derived from the 5126 core promoter, the SGB6 core promoter, the G9 core promoter and the Cauliflower Mosaic Virus 35S core promoter.

25 A particularly preferred core promoter is the 5126 core promoter which comprises the following nucleotide sequence:

5' AGATCTAAGT AAGGTATATA TGTGCATAGT CTCCTAATT
TCTAGATTCA TTCCATATAT ACACGTATCA GAGGATTAAG

30 TTCATCTTCA ACCTCTAGCT GATTGATCTC TGGTATTTAC
AAGTAGAAAGT TGGAGATCGA CTAACTAGAG ACCATAAATG

CACTCTTCC TTCCTTCCTT CCTTCAATT CAAATACCAC
GTGAGAAAGG AAGGAAGGAA GGAAGTTAAG ATTTATGGTG

AAATCAAAGT TGCTTGCCA TG 3' [SEQ ID NO:4].
TTTAGTTCA ACGAAACGGT AC

35 The SGB6 core promoter consists of about 38 nucleotides upstream of the transcriptional start site of

the SGB6 gene. A suitable SGB6 core promoter has the following nucleotide sequence:

5' ATCTCACCCCT ATTAATACCA TGCTGACGAG
TAGAGTGGGA TAATTATGGT ACGACTGCTC

5 CCAATAGC 3' [SEQ ID NO: 5].
GGTTATCG

A suitable core promoter derived from the G9 gene comprises the nucleotide sequence:

10 5' AGATCTCTAT AAAACACGCA GGGACTGGAA AGCGAGATTT
TCTAGAGATA TTTTGTCGCGT CCCTGACCTT TCGCTCTAAA

CACAGCTGAA AGCAGCCAAA ACGCAGAACG TGCAC TGCAT
GTGTCGACTT TCGTCGGTT TCGGTCTTCG ACGTGACGTA

ACATCGAGCT AACTATCTGC AGCCATG 3' [SEQ ID NO:6].
TGTAGCTCGA TTGATAGACG TCGGTAC

15 Suitable core promoters also are provided by functional fragments of 5126, SGB6 and G9 core promoters. Methods for obtaining such functional fragments are described above.

20 The developmental window of avidin gene expression can be extended by using a chimeric regulatory element comprising an anther box from one gene and an anther-specific promoter from a second gene. For example, SGB6 regulatory sequences stimulate gene expression from quartet through mid-uninucleate stages of development, 25 while G9 regulatory sequences stimulate gene expression during meiosis and through the quartet stage of development. Yet the combination of an SGB6 anther box and a G9 promoter stimulates transcription of a foreign gene during meiosis and through the mid-uninucleate stage 30 of development. Thus, various combinations of anther boxes and anther-specific promoters are particularly useful for the present invention.

35 A viral core promoter also may be used. Examples of suitable viral core promoters include a Cauliflower Mosaic Virus (CaMV) core promoter, a Figwort Mosaic Virus core promoter, and the like. Gruber et al., *supra*. Preferably, the viral core promoter is the CaMV 35S core promoter, or a variant thereof.

In order to select transformed cells, the expression vector contains a selectable marker gene, such as a herbicide resistance gene. For example, such genes may confer resistance to phosphinothricine, glyphosate, 5 sulfonylureas, atrazine, or imidazolinone. Preferably, the selectable marker gene is the bar gene or pat gene which encodes phosphinothrin acetyltransferase. The nucleotide sequences of bar genes can be found in Leemans et al., European patent application No. 0-242-246 (1987), 10 and in White et al., *Nucleic Acids Res.* 18: 1062 (1990). Wohlleben et al., *Gene* 70: 25 (1988), disclose the nucleotide sequence of the pat gene. Bar or pat gene expression confers resistance to herbicides such as glufosinate (sold as Basta® and Ignite®, among others) 15 and bialaphos (sold as Herbi-ace® and Liberty®).

The expression vector can contain DNA sequences encoding avidin under the control of a constitutive promoter or an anther-specific promoter, as well as the selectable marker gene under control of a constitutive 20 promoter. Alternatively, the selectable marker gene can be delivered to host cells in a separate selection expression vector by "co-transformation" of embryonic tissue.

B. Restoration of Male Fertility in the F1 Hybrid

25 The above-described methods can be used to produce transgenic male-sterile plants for the production of F1 hybrids in large-scale directed crosses between inbred lines. If all egg cells of the transgenic male-sterile plants do not contain the recombinant avidin gene, then 30 a proportion of F1 hybrids will have a male-fertile phenotype. On the other hand, F1 hybrids will have a male-sterile phenotype if the recombinant avidin gene is present in all egg cells of the transgenic male-sterile plants because sterility induced by ethylene would be 35 dominant. Thus, it might be desirable to use a male fertility restoration system to provide for the production of male-fertile F1 hybrids. Such a fertility

restoration system has particular value in autogamous species when the harvested product is seed.

The commonly proposed approach to restoring fertility in a line of transgenic male-sterile plants requires the 5 production of a second "restorer" line of transgenic plants. For example, a transgenic plant that is male-sterile due to barnase expression would be crossed with a male-fertile plant that expresses the barnase inhibitor, as discussed above. Mariani et al., *Nature* 10 357: 384 (1992).

In the present case, an analogous approach would require the production of a restorer line of transgenic plants that express ribozymes targeted to avidin mRNA or that express antisense avidin. For example, ribozymes 15 can be designed to express endonuclease activity that is directed to a certain target sequence in a mRNA molecule. For example, Steinecke et al., *EMBO J.* 11:1525 (1992), achieved up to 100% inhibition of neomycin phosphotransferase gene expression by ribozymes in 20 tobacco protoplasts. More recently, Perriman et al., *Antisense Res. & Devel.* 3:253 (1993), inhibited chloramphenicol acetyl transferase activity in tobacco protoplasts using a vector that expressed a modified hammerhead ribozyme. In the context of the present 25 invention, avidin mRNA provides the appropriate target RNA molecule for ribozymes.

In a similar approach, fertility can be restored by the use of an expression vector containing a nucleotide sequence that encodes an antisense RNA. The binding of 30 antisense RNA molecules to target mRNA molecules results in hybridization arrest of translation. Paterson, et al., *Proc. Natl. Acad. Sci. USA*, 74:4370 (1987). In the context of the present invention, a suitable antisense RNA molecule would have a sequence that is complementary 35 to avidin mRNA. In a preferred embodiment of the invention, the antisense RNA is under the control of an inducible promoter. Activation of this promoter then allows restoration of male fertility.

In a further alternative approach, expression vectors can be constructed in which an expression vector encodes RNA transcripts capable of promoting RNase P-mediated cleavage of avidin mRNA molecules. According to this 5 approach, an external guide sequence can be constructed for directing the endogenous ribozyme, RNase P, to avidin mRNA, which is subsequently cleaved by the cellular ribozyme. Altman et al., U.S. Patent No. 5,168,053; Yuan et al., *Science* 263: 1269 (1994). Preferably, the 10 external guide sequence comprises a ten to fifteen nucleotide sequence complementary to avidin mRNA, and a 3'-NCCA nucleotide sequence, wherein N is preferably a purine. *Id.* The external guide sequence transcripts bind to the targeted mRNA species by the formation of base 15 pairs between the mRNA and the complementary external guide sequences, thus promoting cleavage of mRNA by RNase P at the nucleotide located at the 5'-side of the base-paired region. *Id.*

In an alternative method for restoring male 20 fertility, transgenic male-sterile plants contain an expression vector that, in addition to a promoter sequence operably linked to an avidin gene, also contains a prokaryotic regulatory element. Transgenic male-fertile plants are produced that express a prokaryotic 25 polypeptide under the control of the promoter. In the F1 hybrids, the prokaryotic polypeptide binds to the prokaryotic regulatory sequence and represses the expression of avidin.

For example, the LexA gene/LexA operator system can 30 be used to regulate gene expression pursuant to the present invention. See U.S. patent No. 4,833,080 ("the '080 patent) and Wang et al., *Mol. Cell. Biol.* 13:1805 (1993). More specifically, the expression vector of the male-sterile plant would contain the LexA operator sequence, while the expression vector of the male-fertile 35 plant would contain the coding sequences of the LexA repressor. In the F1 hybrid, the LexA repressor would

bind to the LexA operator sequence and inhibit transcription of the avidin gene.

LexA operator DNA molecules can be obtained, for example, by synthesizing DNA fragments that contain the well-known LexA operator sequence. See, for example, the 5 '080 patent and Garriga et al., *Mol. Gen. Genet.* 236:125 (1992). The LexA gene may be obtained by synthesizing a DNA molecule encoding the LexA repressor. Gene synthesis techniques are discussed above and LexA gene sequences 10 are described, for example, by Garriga et al., *supra*. Alternatively, DNA molecules encoding the LexA repressor may be obtained from plasmid pRB500, American Type Culture Collection accession No. 67758.

Still another method for restoring fertility utilizes 15 the high affinity of avidin for biotin, by spraying developing plants with a solution of biotin. The biotin solution also comprises a minimum amount of an organic cosolvent such as DMSO to ensure complete solubility of the biotin. Spraying is commenced during the meiotic phase of pollen development, and is repeated at regular 20 intervals until pollen shed is observed. The intervals between spraying will vary between 1 and 7 days. In a preferred embodiment of the invention, spraying is repeated every 3 to 5 days.

Those of skill in the art can readily devise other 25 male fertility restoration strategies using prokaryotic regulatory systems, such as the lac repressor/lac operon system or the trp repressor/trp operon system.

The present invention, thus generally described, will 30 be understood more readily by reference to the following examples, which are provided by way of illustration and are not intended to be limiting of the present invention.

EXAMPLE 1: Preparation of male sterile transgenic corn by high constitutive expression of avidin

A method for formation of transgenic corn plants has been described in European Patent Application No. 0 442 5 174A1 which is hereby incorporated by reference. A brief description of that methodology follows.

PHI5168, a vector carrying the avidin gene under control of the ubiquitin promoter and also containing a PINII terminator sequence, was used to form transgenic 10 corn plants. The structure of PHI5168 is shown in Figure 1. PHI610, an expression vector carrying the bar gene under control of the double 35S promoter, and also carrying a PINII terminator sequence, was cotransformed along with the avidin construct, allowing the selection 15 of transgenic plants by treatment of the transformation mixture with bialaphos. The structure of PHI610 is shown in Figure 2. The two expression vectors were transformed into embryogenic suspension cultures derived from type II embryogenic culture according to the method of Green, 20 et al., Molecular Genetics of Plants and Animals, editors Downey, et al., Academic Press, NY, 20, 147 (1983). The cultures were maintained in Murashige and Skoog ("MS") medium as described in Murashige, et al., Physio. Plant; Vol. 15; pp. 453-497; (1962) supplemented with 2,4- 25 dichlorophenoxyacetic acid (2,4-D) at 2 mg/L and sucrose at 30 g/L. The suspension cultures were passed through a 710 micron sieve 7 days prior to the experiment and the filtrate was maintained in MS medium.

Cells were harvested from the suspension culture by 30 vacuum filtration on a Buchner funnel (Whatman No. 614), and 100 ml (fresh weight) of cells were placed in a 3.3 cm petri plate. The cells were dispersed in 0.5 mL fresh culture medium to form a thin layer of cells. The uncovered petri plate was placed in the sample chamber of 35 a particle gun device manufactured by Biolistics Inc., (Geneva, NY). A vacuum pump was used to reduce the pressure in the chamber to 0.1 atmosphere to reduce

deceleration of the microparticles by air friction. The
cells bombarded with tungsten particles having an average
diameter of about 1.2 microns (GTE Sylvania Precision
Materials Group, Towanda, Pennsylvania). An equal
5 mixture of PHI5168 and PHI610 were loaded onto the
microparticles by adding 5 μ l of a DNA solution (1 μ g of
DNA per 100 lambda) in TE buffer at pH 7.7 to 25 μ l of a
suspension of 50 mg of tungsten particles per ml
distilled water in a 1.5 ml Eppendorf tube. The
10 particles became aggregated and settled in the tube.

Cultures of transformed plant cells containing the
foreign genes were cultivated for 4-8 weeks in 560R
(medium) (N6-based medium with 1 mg/ml bialaphos). This
medium selects for cells that express the bar gene.

15 Embryo formation was then induced in the embryogenic
cultures and the cells germinated into plants. A two
culture medium sequence was used to germinate somatic
embryos observed on callus maintenance medium. Callus
was transferred first to a culture medium (maturation
20 medium) containing 5.0 mg/L indoleacetic acid (IAA) for
10 to 14 days while callus proliferation continued.
Callus loading was at 50 mg per plate to optimize
recovery per unit mass of material.

25 Callus was then transferred from "maturation" medium
to a second culture medium containing a reduced level of
IAA (1 mg/L) compared to the first culture medium.
Cultures are placed in the light at this point.
Germinating somatic embryos were characterized by a green
shoot elongating with a connecting root access. Somatic
30 embryos were then transferred to medium in a culture tube
(150 x 25 mm) for an additional 10-14 days. At this
time, the plants were about 7-10 cm tall, and were of
sufficient size and vigor to be hardened to greenhouse
conditions.

35 To harden regenerated plants, plants to be
transferred to the growth chamber were removed from the

sterile containers and solidified agar medium was rinsed off the roots. The plantlets were placed in a commercial potting mix in a growth chamber with a misting device for maintaining the relative humidity near 100% without excessively wetting the plant roots. After 3-4 weeks in the misting chamber the plants were robust enough for transplantation into field conditions.

Plants in the field were analyzed by observing male sterility. Selected plants were then further analyzed for presence of the avidin gene by PCR and Southern blotting, and for expression of avidin by ELISA, by standard methods.

94 plants were analyzed by PCR, of which 53 were found to be fertile, and 41 sterile. When the fertility of each plant was compared to presence of the avidin gene by PCR, a 98% correlation between presence of the gene and plant sterility was found. 5 plants were analyzed in detail for the presence of the avidin gene by Southern blotting. Three plants were shown to contain the avidin gene by Southern analysis. All three plants had sterility problems. Two plants that did not have the avidin gene were completely fertile. There was thus a 100% correlation between the presence of the avidin gene and sterility problems.

EXAMPLE 2: Use of agrobacterium strains containing a binary vector including a DNA sequence encoding avidin to generate male sterile transgenic soybean plants

A method for forming transgenic soybean plants is that described in U.S. Patent Application Ser. No. 07/920,409 which is hereby incorporated by reference. Soybean (Glycine max) seed, of Pioneer variety 9341, is surface sterilized by exposure to chlorine gas evolved in a glass bell jar. Gas is produced by adding 3.5 ml hydrochloric acid (34 to 37% w/w) to 100 ml sodium hypochlorite (5.25% w/w). Exposure is for 16 to 20 hours

in a container approximately 1 cubic ft in volume. Surface sterilized seed is stored in petri dishes at room temperature. Seed is germinated by plating an 1/10 strength agar solidified medium according to Gambourg (B5
5 basal medium with minimal organics, Sigma Chemical Catalog No. G5893, 0.32 gm/L sucrose; 0.2% weight/volume 2-(N-morpholino) ethanesulfonic acid (MES), (3.0 mM) without plant growth regulators and culturing at 28° with a 16-hour day length and cool white fluorescent
10 illumination of approximately $20 \mu\text{Em}^{-2} \text{ S}^{-1}$. After 3 or 4 days, seed is prepared for co-cultivation. The seed coat is removed and the elongating radical is removed 3 to 4 mm below the cotyledons.

Overnight cultures of Agrobacterium tumefaciens
15 strain LBA4404 harboring a modified binary plasmid containing the avidin gene are grown to log phase in minimal A medium containing tetracycline, 1 $\mu\text{g}/\text{ml}$, and are pooled and an optical density measurement at 550nm is taken. Sufficient volume of the culture is placed in 15
20 mL/conical centrifuge tubes such that upon sedimentation between 1 and 2×10^{10} cells are collected in each tube with 10^9 cells/ml. Sedimentation is by centrifugation at 6,000 $\times g$ for 10 min. After centrifugation, the supernatant is decanted and the tubes are held at room
25 temperature until inoculum is needed, but not longer than 1 hour.

Inoculations are conducted in batches such that each plate of seed is treated with a newly resuspended pellet of Agrobacterium. Bacterial pellets are resuspended
30 individually in 20 ml inoculation medium, containing B5 salts (G5893), 3.2 g/L; sucrose, 2.0% w/v; 6-benzylaminopurine (BAP), 45 μM ; indolebutyric-acid (IBA), 0.5 μM ; acetosyringone (AS), 100 μM ; buffered to pH 5.5 with MES 10 mM. Resuspension is achieved by vortexing.
35 The inoculum is then poured into a petri dish containing prepared seed and the cotyledonary nodes are macerated with a surgical blade. This is accomplished by dividing

seed in half by longitudinal section through the shoot apex, preserving the 2 whole cotyledons. The two halves of the shoot apex are then broken off their respective cotyledons by prying them away with a surgical blade.

5 The cotyledonary node is then macerated with a surgical blade by repeated scoring along the axis of symmetry. Care is taken not to cut entirely through the explant to the axial side. Explants are prepared in roughly 5 minutes and then incubated for 30 minutes at room

10 temperature with bacteria but without agitation. After 30 minutes, the explants are transferred into plates of the same medium solidified with Gelrite (Merck & Company Inc.), 0.2% w/v. Explants are embedded with adaxial side up and leveled with the surface of the medium and

15 cultured at 22°C for 3 days under cool white fluorescent light, approximately $20 \mu\text{Em}^{-2} \text{S}^{-1}$.

After 3 days, the explants are moved to liquid counterselection medium containing B5 salts (G5893), 3.2 g/l; sucrose, 2% w/v; BAP, 5 μM ; IBA, 0.5 μM ; vancomycin, 200 $\mu\text{g}/\text{ml}$; cefotaxime, 500 $\mu\text{g}/\text{ml}$, buffered to pH 5.7 with MES, 3 mM. Explants are washed in each petri dish with constant slow gyratory agitation at room temperature for 4 days. Counterselection medium is replaced 4 times.

The explants are then picked to agarose-solidified selection medium containing B5 salts (G5893), 3.2 g/l; sucrose, 2% w/v; BAP, 5.0 μM ; IBA, 0.5 μM ; kanamycin sulfate, 50 $\mu\text{g}/\text{ml}$; vancomycin, 100 $\mu\text{g}/\text{ml}$; cefotaxime, 30 $\mu\text{g}/\text{ml}$; timentin, 30 $\mu\text{g}/\text{ml}$, buffered to pH 5.7 with MES, 3mM. Selection medium is solidified with Seakem Agarose, 0.3% w/v. The explants are embedded in the medium, adaxial side down and cultured at 28° with a 16 hour day length in cool white fluorescent illumination of 60 to 80 $\mu\text{Em}^{-2} \text{S}^{-1}$.

35 After 2 weeks explants are again washed with liquid medium on the gyratory shaker. The wash is conducted overnight in counterselection medium containing kanamycin

sulfate, 50 $\mu\text{g}/\text{ml}$. The following day, explants are picked to agarose/solidified selection medium. They are embedded in the medium adaxial side down and cultured for another 2 week period.

5 After 1 month on selection medium, transformed tissue is visible as green sectors of regenerating tissue against a background of bleached nonhealthy tissue. Explants without green sectors are discarded and explants with green sectors are transferred to elongation medium
10 containing B5 salts (G5893), 3.2 g/l; sucrose, 2% w/v; IBA, 3.3 μM ; gibberellic acid, 1.7 μM ; vancomycin, 100 $\mu\text{g}/\text{ml}$; cefotaxime, 30 $\mu\text{g}/\text{ml}$; and timentin, 30 $\mu\text{g}/\text{ml}$, buffered to pH 5.7 with MES, 3 mM. Elongation medium is solidified with Gelrite, 0.2% w/v. The green sectors are
15 embedded adaxial side up and cultured as before. Culture is continued on this medium with transfers to fresh plates every two weeks. When shoots are 0.5 cm in length they are excised at the base and placed in rooting medium in 13 x 100 ml test tubes. Rooting medium consists of B5
20 salts (G5893), 3.2 g/l; sucrose, 15 g/l; nicotinic acid, 20 μM ; pyroglutamic acid (PGA), 900 mg/L and IBA, 10 μM . The rooting medium is buffered to pH 5.7 with MES, 3 mM and solidified with Gelrite at 0.2% w/v. After 10 days, the shoots are transferred to the same medium without IBA
25 or PGA. Shoots are rooted and held in these tubes under the same environmental conditions as before.

When a root system is well established the plantlet is transferred to sterile soil. Temperature, photoperiod and light intensity remain the same as before.

30 The expression of avidin in transgenic soybean plants is confirmed and quantitated using ELISA, and presence of the gene is confirmed by PCR and Southern blotting. Stability of expression can be evaluated by these same methods over successive generations. Sterility problems
35 are found to correlated with expression of avidin in the soybeans.

EXAMPLE 3: Preparation of male sterile sunflower plants by expression of avidin

5 An expression cassette encoding avidin is used to generate transgenic sunflower plants and seeds. The DNA sequence coding for avidin is inserted into an expression cassette under control of the ubiquitin promoter.

10 This expression cassette is then subcloned into a binary vector such as PHI 5765 using the EcoR1 site. The binary vector is then transferred into an Agrobacterium tumefaciens helper strain.

15 Sunflower plants are transformed with Agrobacterium strain LBA4404 after microparticle bombardment as described by Bidney, et al., Plant Mol. Bio.; Vol. 18; p. 301; 1992. Briefly, seeds of Pioneer Sunflower Line SMF-3 are dehulled and surface sterilized. The seeds are imbibed in the dark at 26°C for 18 hours on filter paper moistened with water. The cotyledons and root radical are removed and meristem explants cultured on 374BGA medium (MS salts, Shephard vitamins, 40 mg/L adenine sulfate, 3% sucrose, 0.8% phytagar pH 5.6 plus 0.5 mg/L of BAP, 0.25 mg/L, IAA and 0.1 mg/L GA). Twenty-four hours later, the primary leaves are removed to expose the apical meristem and the explants are placed with the apical dome facing upward in a 2 cm circle in the center 20 of a 60 mm by 20 mm petri plate containing water agar. The explants are bombarded twice with tungsten particles suspended in TE buffer or with particles associated with an expression plasmid containing the avidin gene. The meristem explants are co-cultured on 374BGA medium in the 25 light at 26°C for an additional 72 hours of co-culture.

30 Agrobacterium treated meristems are transferred following the 72 hour co-culture period to medium 374 (374BGA with 1% sucrose and no BAP, IAA or GA₃) and supplemented with 250 µg/ml cefotaxime. The plantlets 35 are allowed to develop for an additional 2 weeks under 16 hour day and 26°C incubation conditions to green or

bleach. Plantlets are transferred to medium containing kanamycin and allowed to grow. The presence of avidin in the plants is confirmed and quantitated as described in Example 2. The presence of male sterility is found to 5 correlate with expression of avidin by the plants.

Although the foregoing refers to particular preferred embodiments, it will be understood that the present invention is not so limited. It will occur to those of ordinary skill in the art that various modifications may 10 be made to the disclosed embodiments and that such modifications are intended to be within the scope of the present invention, which is defined by the following claims. All publications and patent applications mentioned in this specification are indicative of the 15 level of skill of those in the art to which the invention pertains.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: PIONEER HI-BRED INTERNATIONAL, INC.
- (B) STREET: 700 CAPITAL SQUARE, 400 LOCUST STREET
- (C) CITY: DES MOINES
- (D) STATE: IOWA
- (E) COUNTRY: UNITED STATES OF AMERICA
- (F) POSTAL CODE: 50309

(ii) TITLE OF INVENTION: INDUCTION OF MALE STERILITY IN PLANTS BY
EXPRESSION OF HIGH LEVELS OF AVIDIN

(iii) NUMBER OF SEQUENCES: 6

(iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: Foley & Lardner
- (B) STREET: 3000 K Street, N.W., Suite 500
- (C) CITY: Washington
- (D) STATE: D.C.
- (E) COUNTRY: USA
- (F) ZIP: 20007-5109

(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER: UNASSIGNED
- (B) FILING DATE:

(vii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 08/475,582
- (B) FILING DATE: 07-JUN-1995

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: BENT, Stephen A.
- (B) REGISTRATION NUMBER: 29,768
- (C) REFERENCE/DOCKET NUMBER: 33229/403/PIHI

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: (202) 672-5300
- (B) TELEFAX: (202) 672-5399
- (C) TELEX: 904136

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 64 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCGGCCGCGGG ATCCGCTCAT CTGCTCGGTA CCAACCAGCC TTTCTTATTAA CCATGCACAG

60

ATCT

64

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 94 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ACAGTTCACT AGATATGCAT GATCTTTAAC AATTGCTGCT GGATTGTGCG GTTTCTTTG 60

GCACAAATGG CATGAACAGA GTAATCCGGG ACGC 94

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 136 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GCGGCCGCGG ATCCTGGCTG GATGAAACCG ATGCGAGAGA AGAAAAAAA ATTGTTGCAT 60

GTAGTTGGCG CCTGTCACCC AACCAAACCA GTAGTTGAGG CACGCCCTGT TTGCTCACGA 120

TCACGAACGT AGATCT 136

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 142 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- 35 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AGATCTAAGT AAGGTATATA TGTGCATAGT CTCCTAATTCA TTCACTTTCA ACCTCTAGCT	60
GATTGATCTC TGGTATTTAC CACTCTTCC TTCCCTTCCTT CCTTCAATTCA TAAATACCAC	120
AAATCAAAGT TGCTTTGCCA TG	142

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 38 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATCTCACCT ATTATACCA TGCTGACGAG CCAATAGC	38
---	----

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 107 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AGATCTCTAT AAAACACGCA GGGACTGGAA AGCGAGATTT CACAGCTGAA AGCAGCCAAA	60
ACGCAGAAGC TGCCTGCAT ACATCGAGCT AACTATCTGC AGCCATG	107

-36-

What Is Claimed Is:

1. An isolated DNA molecule comprising a nucleotide sequence encoding avidin operably linked to a plant promoter sequence.
2. An expression vector comprising the isolated DNA molecule of claim 1.
3. An expression according to claim 2, wherein said plant promoter sequence is a constitutive promoter.
4. An isolated DNA molecule according to claim 3, wherein said constitutive promoter sequence is a ubiquitin promoter sequence.
5. A transgenic plant, wherein said plant comprises a heterologous nucleotide sequence comprising an avidin gene, wherein said heterologous nucleotide sequence increases the concentration of avidin in the tissues of the plant, and whereby said increased concentration of avidin causes said plant to be male sterile.
6. A transgenic plant according to claim 5, wherein said plant is a corn plant.
7. A transgenic plant according to claim 5, wherein said plant is a soybean plant.
8. A transgenic plant according to claim 5, wherein said plant is a sunflower plant.
9. A transgenic plant according to claim 5, wherein said heterologous nucleotide sequence further comprises a constitutive promoter sequence.
10. A transgenic plant according to claim 9, wherein said constitutive promoter sequence is a ubiquitin promoter sequence.

-37-

11. A method of producing a transgenic male-sterile plant, comprising introducing into plant cells an expression vector comprising a promoter and an avidin gene, wherein said promoter controls the expression of said avidin gene, and whereby said avidin gene expression causes male sterility.

12. The method of claim 11, further comprising regenerating a transgenic plant from said plant cells.

13. The method of claim 11, wherein said promoter comprises a ubiquitin promoter.

14. A method of using an avidin gene to produce a male-fertile hybrid plant, comprising the steps of:

- (a) producing a first parent male-sterile plant comprising a DNA molecule according to claim 1, wherein the expression of avidin causes male sterility;
- (b) producing a second transgenic parent plant expressing a second foreign gene; and
- (c) cross-fertilizing said first parent with said second parent to produce a hybrid plant,

wherein said hybrid plant expresses said second foreign gene, and wherein the product of said second foreign gene reduces expression of avidin in said hybrid plant, thereby producing a male-fertile hybrid plant.

15. The method of claim 14, wherein said second foreign gene is selected from the group consisting of an antisense gene, a ribozyme gene and an external guide sequence gene.

16. The method of claim 15, wherein said antisense gene comprises avidin mRNA sequences.

17. The method of claim 16, wherein said ribozyme gene comprises avidin mRNA sequences.

18. The method of claim 16, wherein said external guide sequence gene comprises avidin mRNA sequences.

-38-

19. The method of claim 14, wherein said DNA molecule of said first parent plant further comprises a LexA operator which is operatively linked to said promoter, and wherein said second foreign gene is the LexA repressor gene.

20. The expression vector of claim 2, wherein said promoter sequence is an anther-specific promoter sequence comprising an anther box selected from the group consisting of:

- (a) a DNA molecule having the nucleotide sequence of SEQ ID NO:1;
- (b) a DNA molecule having the nucleotide sequence of SEQ ID NO:2;
- (c) a DNA molecule having the nucleotide sequence of SEQ ID NO:3; and
- (d) a functional fragment of (a), (b) or (c).

21. The expression vector of claim 20, wherein said anther box has the nucleotide sequence of SEQ ID NO:1, and wherein said anther-specific promoter further comprises a core promoter selected from the group consisting of:

- (a) CaMV 35S core promoter;
- (b) a DNA molecule having the nucleotide sequence of SEQ ID NO:4;
- (c) a DNA molecule having the nucleotide sequence of SEQ ID NO:5;
- (d) a DNA molecule having the nucleotide sequence of SEQ ID NO:6; and
- (e) a functional fragment of (b), (c) or (d).

22. The expression vector of claim 20, wherein said anther box has the nucleotide sequence of SEQ ID NO:2, and wherein said anther-specific promoter further comprises a core promoter selected from the group consisting of:

- (a) CaMV 35S core promoter;

-39-

- (b) a DNA molecule having the nucleotide sequence of SEQ ID NO:4;
- (c) a DNA molecule having the nucleotide sequence of SEQ ID NO:5;
- (d) a DNA molecule having the nucleotide sequence of SEQ ID NO:6; and
- (e) a functional fragment of (b), (c) or (d).

23. The expression vector of claim 20, wherein said anther box has the nucleotide sequence of SEQ ID NO:6, and wherein said anther-specific promoter further comprises a core promoter selected from the group consisting of:

- (a) CaMV 35S core promoter;
- (b) a DNA molecule having the nucleotide sequence of SEQ ID NO:4;
- (c) a DNA molecule having the nucleotide sequence of SEQ ID NO:5;
- (d) a DNA molecule having the nucleotide sequence of SEQ ID NO:6; and
- (e) a functional fragment of (b), (c) or (d).

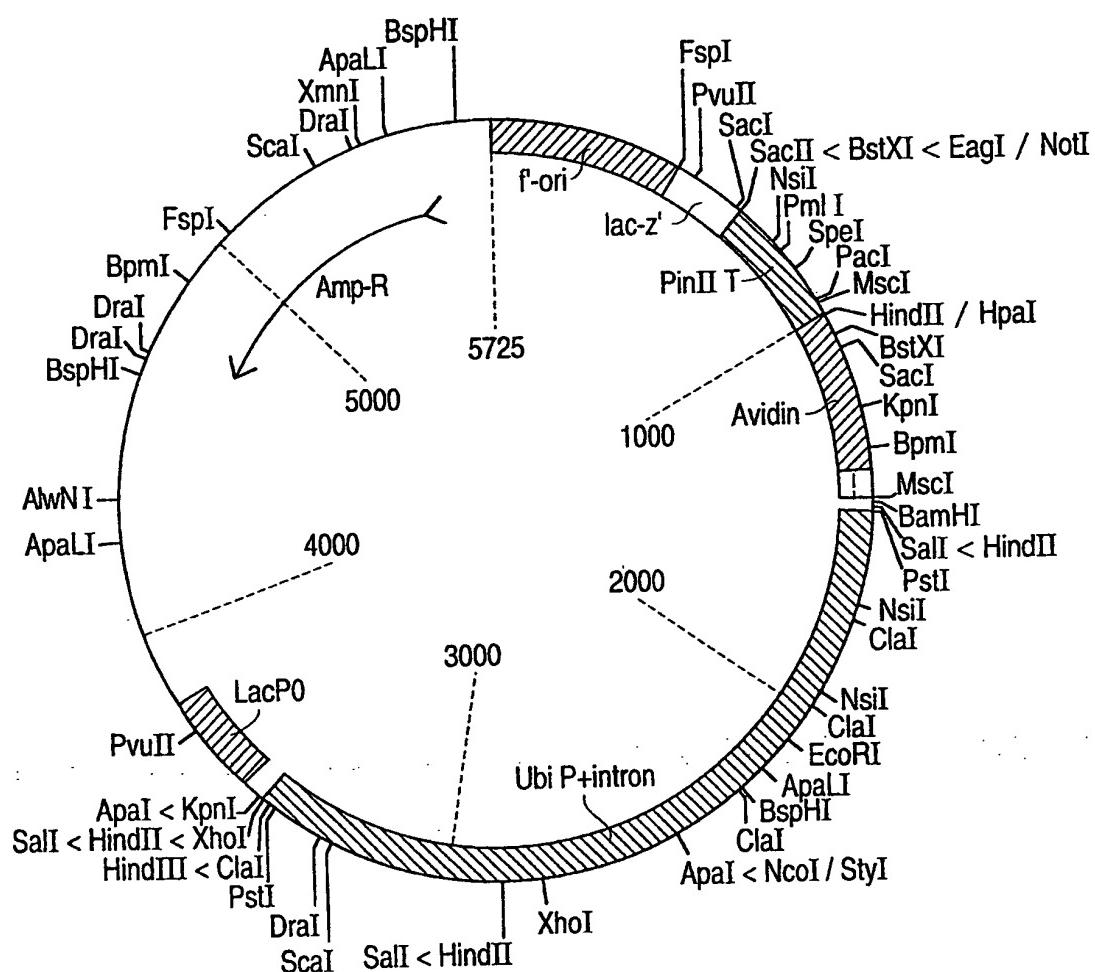
24. A method for restoring fertility in a plant rendered male sterile by expression of avidin, comprising spraying said plant with a solution of biotin.

25. The method of claim 11, wherein said promoter comprises an inducible promoter.

26. The method of claim 15, wherein said antisense gene is operably linked to an inducible promoter.

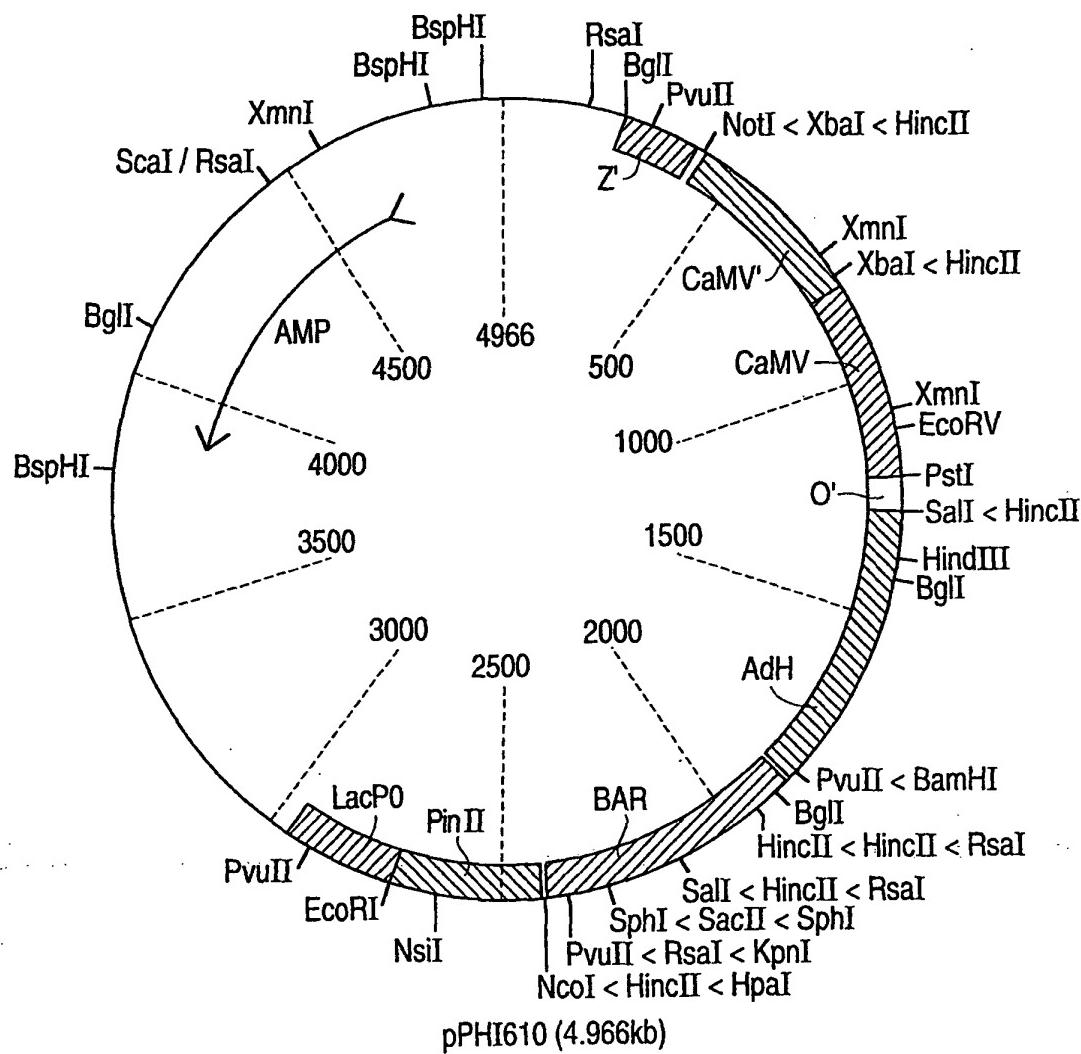
1/2

FIG. 1



2/2

FIG. 2



INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 96/08583

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C12N15/82 C12N15/12 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 00992 (PIONEER HI BRED INT ;US GOVERNMENT (US)) 20 January 1994 see the whole document ---	1-3
X	JOINT ANNUAL MEETING OF THE AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS AND THE CANADIAN SOCIETY OF PLANT PHYSIOLOGISTS , MINNEAPOLIS, MINNESOTA, USA, JULY 31-AUGUST 4, 1993. PLANT PHYSIOL (ROCKV) 102 (1 SUPPL.). 45., XP002017747 GUAN X ET AL: "EXPRESSION OF STREPTAVIDIN GENE IN TOBACCO AND ITS EFFECTS ON BACTERIA." see abstract 234 ---	1-3
P,X	WO,A,96 16182 (PIONEER HI BRED INT) 30 May 1996 see page 32, line 3 - line 6 ---	1-3 -/-

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

1

Date of the actual completion of the international search

6 November 1996

Date of mailing of the international search report

15.11.96

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl.
 Fax (+ 31-70) 340-3016

Authorized officer

Maddox, A

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 96/08583

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,86 02077 (MEADE HARRY M;GARWIN JEFFREY L; BIOGEN NV) 10 April 1986 see claim 9 ---	1-3
A	WO,A,94 21804 (PIONEER HI BRED INT ;NEILL JOHN D (US); PIERCE DOROTHY A (US); CIG) 29 September 1994 see the whole document -----	1-26

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 96/08583

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9400992	20-01-94	AU-A-	4671293	31-01-94
WO-A-9616182	30-05-96	AU-A-	4194496	17-06-96
WO-A-8602077	10-04-86	DE-A- EP-A- JP-A- JP-T- US-A- US-A-	3583940 0198015 8228791 62500700 5168049 5272254	02-10-91 22-10-86 10-09-96 26-03-87 01-12-92 21-12-93
WO-A-9421804	29-09-94	AU-A- BR-A- CA-A- DE-T- HU-A- JP-T- NL-T- PL-A-	6355194 9405950 2158584 4491714 73336 8507691 9420020 310702	11-10-94 19-12-95 29-09-94 27-06-96 29-07-96 20-08-96 01-05-96 27-12-95